(54) Title: WATER-SOLUBLE FORMULATIONS OF RESVERATROL AND USES THEREOF

![Diagram of Resveratrol](image-url)

S-OOR: 3-0-Glucuronidated Resveratrol

Fig. 3

(57) Abstract: An aqueous solution containing trans-3',4',5-trihydroxystilbene (resveratrol), water, and an effective amount of a solubilizer containing poloxamer 334 (Pluronic® 104), wherein the solubilizer renders the trans-3',4',5-trihydroxystilbene water soluble, is disclosed. The solution can be used as a medicament for the treatment of cancer.
WATER-SOLUBLE FORMULATIONS OF RESVERATROL AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Patent Application No. 61/15,568, filed on November 18, 2008, which is incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with United States government support awarded by the following agency: National Institutes of Health - Grant No. CAI 03653. The United States government has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0003] Despite increasing awareness, prevention programs, screening, testing, improved treatments and regimen, aggressive interventions and follow-up, cancer remains a leading cause of death in many countries. According to the American Cancer Society's Global Cancer Facts and Figures for 2007, deaths due to cancer will increase from 7.6 million in 2007 to 17.5 million in 2050. Numerous chemotherapeutic drugs have been developed but few have tumor specificity, most cannot prevent secondary tumors and recurrence, many have limited efficacy due to drug-resistance and almost all are associated with severe, dose-limiting, systemic toxicities. Other than some early-stage pediatric cancers and select adult cancers, progress in effective treatments has been modest. Successful chemoprevention should be the long-term goal but at present there is an urgent need for the development of relatively non-toxic therapeutic agents that can improve cancer survival without destroying the quality of life. This is particularly relevant to aggressive childhood cancers such as advanced stages of neuroblastoma where currently available treatments are often ineffective and have serious side-effects such as acute damage, toxicity and increased occurrence of secondary tumors (Ebb et al, 2001).

[0004] Extensive pre-clinical and clinical studies have been carried out on the chemopreventive benefits of resveratrol (trans-3,4',5-trihydroxystilbene). A naturally occurring polyphenol found in grapes, berries and peanuts; resveratrol was also discovered to be the active ingredient in the Asian traditional medicine derived from the Japanese knotweed, Polygonum cuspidatum. Mechanistic and pre-clinical studies on retinoblastoma, neuroblastoma, uveal melanoma and breast cancer suggest that at supplemental concentrations exceeding those obtained from a normal diet or moderate

In vivo studies in animals and humans indicate that resveratrol is poorly absorbed from the gastrointestinal tract and undergoes extensive first-pass metabolism, mainly glucuronidation and sulfation, in the gut and liver, leading to trace amounts of the compound in the serum (De Santi et al, 2000, de Santi et al, 2000, Marier et al, 2002, Miksits et al, 2005, Wenzel and Somoza, 2005, Yu et al, 2002). The demonstrated efficacy of resveratrol in spite of low bioavailability remains a conundrum and the contribution of the metabolites to resveratrol’s efficacy has thus far not been determined (Asensi et al, 2002, Gescher and Steward, 2003, Kundu and Surh, 2008, Walle et al, 2004). There are suggestions that there might be dose- and species-dependent differences in metabolite profiles (Abd El-Mohsen et al, 2006, Boocock et al, 2007, Meng et al, 2004). Our previous studies in neuroblastoma indicate that despite low bioavailability, resveratrol is effective at inhibiting tumor growth, and if elevated levels can be achieved, widespread tumor cell death leading to tumor regression can be achieved (van Ginkel et al, 2007). Poor gastrointestinal uptake requires that alternative routes of delivery that allow for direct infusion of resveratrol into the bloodstream are necessary for achieving elevated serum drug levels. However, the poor solubility of resveratrol in water (<50 µg/ml) requires the use of oil-based formulations which are limited largely to oral administration. Thus, there is a need in the art for novel non-toxic aqueous formulations of resveratrol wherein the solubility of resveratrol is substantially greater than in currently known formulations.

SUMMARY OF THE INVENTION

Accordingly, in a first aspect, the invention encompasses an aqueous solution in which rm–3,4,5-trihydroxystilbene (resveratrol) is rendered water soluble. The solution contains trans-3,4',5-trihydroxystilbene, water, and an effective amount of a solubilizer containing poloxamer 334. The solubilizer acts to render the traMs-3,4',5-trihydroxystilbene water soluble.

Preferably, the poloxamer 334 makes up between 5% and 20% of the solution by weight; more preferably, the poloxamer 334 makes up about 10% of the solution by weight. In some embodiments, the solution may additionally contain ethanol.

In the present invention, the use of poloxamer 334 as a solubilizer produces a surprisingly high solubility for traMs-3,4',5-trihydroxystilbene in water. In certain preferred embodiments, the
concentration of /røn.s-3,4',5-trihydroxystilbene solubilized in the solution is between 20 mg/mL and 30 mg/mL.

[0009] The solution as described above may be used in the treatment of cancer, including without limitation in the treatment of neuroblastoma, breast cancer, cutaneous melanoma or uveal melanoma. Thus, in a second aspect, the invention encompasses a method of treating cancer. Such a method includes the step of administering to a patient having cancer a solution as described above. The amount of solution administered is an amount sufficient to reduce cancer symptoms.

[00010] In some embodiments of the method, a patient having a specific type of cancer is treated. Specific types of cancer that can be treated by the method include without limitation neuroblastoma, breast cancer, cutaneous melanoma, and uveal melanoma. The solution may be administered to the patient using a variety of methods known in the art. Non-limiting examples of methods for administering the solution include intravenous injection, intraperitoneal injection, intratumor injection, peri-tumor injection or oral administration.

[00011] In a third aspect, the invention encompasses a method of making an aqueous solution wherein /røns-3,4',5-trihydroxystilbene is rendered water soluble. The method includes the step of mixing /rø«.s-3,4',5-trihydroxystilbene, water, and poloxamer 334 to make a solution. Preferably, the amount of poloxamer 334 mixed in to make the solution is from 5-20% of the total mixture by weight; more preferably, the amount of poloxamer 334 mixed in to make the solution is about 10% of the total mixture by weight.

[00012] In certain embodiments of the method, ethanol is added and mixed with the trans-3,4\=
trihydroxystilbene, water, and poloxamer 334 to make the solution. In some such embodiments, the step of mixing the required ingredients make the solution is further divided into two separate mixing sub-steps. The ethanol and /rø«.a-5,3',4,5-trihydroxystilbene are mixed in one sub-step to make a first solution, and the water and the poloxamer 334 are mixed in another sub-step to make a second solution. The resulting first and second solutions are then mixed together to make the aqueous solution of the invention.

[00013] In certain preferred embodiments, to maximize the amount of trans-3,4\=
trihydroxystilbene solubilized in the solution, the amount of /rø«.a-5,3',4,5-trihydroxystilbene added to the mixture is in excess of 30 mg /rø«.s-3,4',5-trihydroxystilbene per mL of mixture. In some such embodiments, the method may include the additional step of centrifuging the mixture and removing the solution as the supernatant liquid. The method may additionally include the step of passing the solution through a filter, preferably a filter having a pore size of about 0.2µm.
Because the solution can be used to treat a patient having cancer, the method of making the solution as described above also encompasses a method of making a medicament solution for the treatment of cancer. Such a method includes the step of mixing resveratrol 3',4',5-trihydroxystilbene, water, and poloxamer 334 to make a solution, wherein the solution is effective for reducing cancer symptoms when administered to a patient having cancer. In some embodiments, the method is used for making a medicament effective against a specific type of cancer, including without limitation neuroblastoma, breast cancer, retinoblastoma, cutaneous melanoma, or uveal melanoma. The method further encompasses all of the various embodiments of the method of making the aqueous solution as described above.

Other objects, features and advantages of the present invention will become apparent after review of the specification, claims and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Particle size distribution in R-P104. The size distribution of micellar particles was determined by dynamic light scattering. The mean particle size was 65nm +/- 30.7nm.

Figure 2. Resveratrol decreases cell viability of SK-N-AS cells. R-P104 and stock resveratrol in DMSO (Res, 200mM) were diluted in cell culture media to obtain resveratrol concentrations of 50 and 100μM. Similar dilutions were carried out with the vehicles (0.01% P104, DMSO). Fluorescence intensity was measured as a function of resveratrol concentration and time after drug addition and plotted to indicate changes in cell viability compared to untreated control cells. Measurements were done in triplicate and the experiment repeated twice.

Figure 3. The chemical structural formula for the 3-O-glucuronidated resveratrol derivative: 3-OGR.

Figure 4. The chemical structural formula for the 4'-O-glucuronidated resveratrol derivative: 4'-OGR.

Figure 5. In vitro efficacy of glucuronidated metabolites of resveratrol. The viability of SK-N-AS cells in the presence of 4'-OGR (labeled in Figure 5 as 4'-ORG) and 3-OGR (labeled in Figure 5 as 3-ORG) were compared with equivalent resveratrol (Res) concentrations over 4 days. Stock solutions in DMSO were appropriately diluted in cell culture media to obtain desired drug concentrations. Fluorescence intensity was measured as a function of concentration and time after drug addition and plotted to indicate changes in cell viability compared to untreated control cells. Measurements were done in triplicate and the experiment repeated twice.
Figure 6. Inhibition of neuroblastoma tumor growth. A) Tumor growth kinetics during R-P 104 treatment course of SK-N-AS xenograft models by peri-tumor injections. Plotted is the change in average tumor volume in R-P104-treated and vehicle-treated groups of mice. Each arrow indicates a single 200 µL dose. B) Photographs of three representative mice from vehicle (left panel) and R-P 104 (right panel) treated groups after five peri-tumor injections.

Figure 7. Neuroblastoma tumor histology. H&E-stained paraffin sections of tumors removed at the end of study from mice after five injections of A) vehicle-treated peri-tumor; B) R-P104-treated peri-tumor; C) vehicle-treated intra-tumor; D) R-P104-treated intra-tumor injections.

DETAILED DESCRIPTION OF THE INVENTION

Definitions. Throughout this specification, the following definitions shall apply.

"DMSO" means dimethylsulfoxide.

"FDA" means U.S. Food and Drug Administration.

"H&E" means Hematoxylin-Eosin.

"HPLC" means high-performance liquid chromatography.

"IP" means intraperitoneal.

"Poloxamer" or "Pluronic®" mean polyoxypropylene-polyoxyethylene non-ionic block copolymers having the structure shown below composed of two blocks or chains of hydrophilic polyoxyethylene flanking a single block of hydrophobic polyoxypropylene. In the structure below, 'a' represents the polyoxyethylene portion and 'b' represents polyoxypropylene portion.

\[
\text{CH}_3 \quad H\text{O}-\text{CH}_2\text{-CH}_2\text{-O}\{\text{CH}_2\text{-CH}_2\text{-O}\}_{5}\text{-f-CH}_2\text{-C}_\text{H} \text{-Ol}_{5}\text{-f-CH}_2\text{-CB}_2\text{-O}\}_{10}\text{-H}
\]

Because the lengths of the polymer blocks can be customized, many different poloxamers exist that have somewhat different properties. For the generic term "poloxamer," these copolymers are named with the letter "P" (for poloxamer) followed by three digits, the first two digits x 100 giving the approximate molecular mass of the polyoxypropylene core, and the last digit x 10 giving the approximate percentage polyoxyethylene content (e.g., P334 is a poloxamer with a polyoxypropylene molecular mass of approximately 3,300 g/mol and a 40% polyoxyethylene content).
For the Pluronic® trade name (proprietary to BASF, Parsippany, NJ), coding of these copolymers starts with a letter to define its physical form at room temperature (L = liquid, P = paste, F = flake (solid)) followed by two or three digits, The first digit (two digits in a three-digit number) in the numerical designation, multiplied by 300, indicates the approximate molecular weight of the hydrophobe. The last digit x 10 gives the approximate percentage polyoxyethylene content. The hydrophilicity of the Pluronic® increases with increasing polyoxyethylene content. In the studies outlined in the Example below, we picked the following Pluronics: one liquid (L61) with 10% polyoxyethylene content, one paste (P104) with 40% polyoxyethylene and one solid (F127) with 70% polyoxyethylene. The molecular weights of the polyoxypropylene moiety of the polymers ranged from approximately 1800 (L61) to approximately 3000 (P104) to approximately 3600 (F127).

"Poloxamer 334," "Pluronic® 104," or "P104" mean the poloxamer sold by BASF (Parsippany, NJ) under the trade name Pluronic® 104.

"IV" means intravenous.

"R-P 104" means the solution of the present invention comprising both resveratrol (R) and poloxamer 334 (P104).

"Resveratrol" means the compound having the following chemical structure. Resveratrol is also known as trøHs-3,4',5-trihydroxystilbene.

"SD" means standard deviation;

"SE" means standard error.

"Water soluble" means that the maximum amount of the given solute that can dissolve in a water solution is greater than 10 milligrams solute per milliliter solution.

Preferred Embodiments of the Invention. In one aspect, the invention encompasses an aqueous solution of trøHs-3,4',5-trihydroxystilbene (resveratrol). The solution contains an effective
amount of poloxamer 334 (P 104), which acts as a solubilizer that renders the trans-3,4',5-trihydroxystilbene water soluble. In certain embodiments, the solution may also contain ethanol as a co-solubilizer.

[00040] Preferably, the solution contains between 5% and 20% poloxamer 334 by weight; more preferably about 10% poloxamer 334 by weight. In certain preferred embodiments, the concentration of trans-3,4',5-trihydroxystilbene in the solution is greater than 20 mg/mL; more preferably, the concentration of trans-3,4',5-trihydroxystilbene is between 20 and 30 mg/mL.

[00041] In certain embodiments, the aqueous solution is for use in treating cancer. Cancers that could be treated with the solution of the invention include without limitation neuroblastoma, breast cancer, retinoblastoma, cutaneous melanoma, or uveal melanoma.

[00042] In a second aspect, the invention is a method of treating cancer by administering an effective amount of the solution described above to a patient having cancer, wherein the amount of solution administered is sufficient to reduce the patient's cancer symptoms. Human cancers which may be treated by the present invention include without limitation neuroblastoma, breast cancer, retinoblastoma, cutaneous melanoma or uveal melanoma. "Treating" means that the solution produces an observable decrease in cancer symptoms. In certain embodiments of the method, the solution is administered by intravenous injection, intraperitoneal injection, intratumor injection, peritumor injection, or orally.

[00043] In a third aspect, the invention encompasses methods of making an aqueous solution of trans-5,3,4',5-trihydroxystilbene solution as described above. The methods include the step of mixing trans-5,3,4',5-trihydroxystilbene, water, and poloxamer 334 to make a solution. Preferably, the amount of poloxamer 334 mixed in to make the solution is from 5-20% of the total mixture by weight; more preferably, the amount of poloxamer 334 mixed in to make the solution is about 10% of the total mixture by weight. In certain embodiments, ethanol may also be added to the mixture.

In some such embodiments, before all the components of the solution are mixed together, the ethanol is mixed with the trans-5,3,4',5-trihydroxystilbene and the poloxamer 334 is separately mixed with the water.

[00044] In certain preferred embodiments, the amount of trans-5,3,4',5-trihydroxystilbene added to the mixture is in excess of 30 mg trans-3,4',5-trihydroxystilbene per mL of mixture, and the resulting solubilized concentration of trans-3,4',5-trihydroxystilbene in the resulting solution is between 20 and 30 mg/mL. In some such embodiments, the solution is separated from the excess trans-5,3,4',5-trihydroxystilbene by the additional step of centrifuging the mixture and removing the
solution as the supernatant liquid. The method may also include the additional step of passing the solution through a filter, preferably a filter having a pore size of about 0.2μm.

[00045] In some embodiments, the method described above can be used in making a medicament for the treatment of cancer. Cancers that could be treated by a medicament made by the method of the invention include without limitation neuroblastoma, breast cancer, retinoblastoma, cutaneous melanoma or uveal melanoma.

[00046] While this invention has been described in conjunction with the various exemplary embodiments outlined in the Example below, various alternatives, modifications, variations, improvements and/or substantial equivalents, whether known or that are or may be presently unforeseen, may become apparent to those having at least ordinary skill in the art.

EXAMPLE

[00047] In general, purified resveratrol has shown potential as a non-toxic anti-cancer agent. However, poor water solubility has limited its administration to oral delivery. Even high dose administration results in poor uptake and rapid clearance through conversion to water-soluble metabolites. The novel aqueous solution of the present invention, R-P 104, uses the block copolymer Pluronic® P 104 to make a water-soluble micelle with resveratrol. In this Example, we demonstrate the successful production of R-P 104, providing an injectable aqueous solution containing >20mg/mL solubilized resveratrol. We also demonstrate that intravenous delivery of the R-P 104 solution increased total peak serum resveratrol concentrations to >100μM with a correspondingly elevated bioavailability of free resveratrol.

[00048] Previously, the in vivo effects of resveratrol treatments have been attributed to both free and modified resveratrol. By chemically synthesizing and testing the two mono-glucuronidated metabolites, we further demonstrate that these metabolites are ineffective against tumor cells in culture. This suggests that the anti-cancer effects might be enhanced with increased levels of unmodified resveratrol, as obtained with R-P104. Direct peri-tumor injections of R-P104 in a subcutaneous neuroblastoma mouse model resulted in 80% tumor inhibition. Low toxicity of R-P 104 made it possible to shrink the tumor further using intra-tumor injections to achieve approximately 90% tumor cell death within two weeks. These findings suggest that solubilizing resveratrol with a block copolymer will provide more efficacious and versatile options for resveratrol delivery.

[00049] In sum, we now report the development of a novel, water-soluble active micelle of resveratrol using a block co-polymer, Pluronic® P 104. We have shown that this formulation (R-
P104) boosts peak serum concentrations of both free and metabolized resveratrol when delivered intravenously. Further, chemically synthesized mono-glucuronidated derivatives were tested for anti-tumor activity and found to be inactive, revealing the importance of enhancing serum levels of free resveratrol to improve potency. *In vivo* studies in an animal model of human neuroblastoma resulted in tumor inhibition demonstrating the efficacy of this formulation and the potential for its development into an effective, non-toxic chemotherapeutic agent.

**Materials and Methods:**

**Materials.** Purified resveratrol was purchased from Cayman Chemical (Michigan, USA). All other chemicals were of reagent grade. Pluronic® P104, F127 and L62 were kindly donated by BASF (BASF, Mount Olive, NJ, USA). Athymic nu/nu mice were purchased from Harlan Sprague Dawley Inc. (Indianapolis, USA). Animals were housed in a pathogen-free isolation facility. All animal care and treatment protocols were in compliance with the guidelines and approved by the University of Wisconsin- Madison Animal Care and Use Committee.

**Cell culture.** SK-N-AS neuroblastoma cell lines were grown as adherent cells at 37°C, 5% CO₂ in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 10 mmol/L HEPES, and 1% penicillin-streptomycin-amphotericin B (Sigma, St. Louis, MO, USA).

**Resveratrol solubility.** Excess resveratrol was added to aqueous solutions of various surfactants in the presence of either dimethyl sulfoxide (DMSO) or ethanol. The resulting solutions were centrifuged at 14,000rpm for 5 min. at room temperature and the drug concentration in the supernatant was determined using high-performance liquid chromatography (HPLC) after the appropriate dilution.

**Resveratrol formulation containing P104 (R-P104).** 200µl ethanol (2%) was added to 20 mg resveratrol, then dissolved in 10 mL of a 10% P104 aqueous solution, filtered through 0.2µm syringe filter to obtain a clear pale yellow solution. The concentration of resveratrol in the solution was quantified by HPLC prior to use.

**HPLC Analytical Method.** Samples were analyzed by reverse-phase HPLC (van Ginkel *et al.*, 2007) using a Gemini C6-phenyl column (4.5 x 250 mm) with 5 micron particle size (Phenomenex, Torrance, CA, USA). The mobile phase consisted of a 40:60 mixture of solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.09% trifluoroacetic acid in 90% acetonitrile - water) pumped at 1 mL/min. The eluent was monitored at 306 nm. Calibration curves were obtained
using resveratrol standard solutions (0-2000 pmoles) and found to be linear with a correlation coefficient greater than 0.99.

[00056] **Particle size measurement.** Particle sizes were determined using dynamic light scattering with the NICOMP ZLS380 particle sizer (Particle Sizing Systems, Santa Barbara, CA, USA) equipped with a 639 nm laser at a fixed angle of 90°. Data was acquired to have greater than 100 k counts in channel 1. Particle sizes were expressed as volume-weighted diameters.

[00057] **In vitro cell viability.** Cells were grown in 96-well microtiter plates for 2 days. Drug treatments were started and corresponding treatment media were replenished after two days. 1 to 4 days later Cell Titer Blue reagent was added according to the manufacturer's instructions (Promega, Madison, WI, USA). Fluorescence was measured at excitation/emission wavelengths of 560/590 nm, using a fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA). IC₅₀ values were determined based on fluorescence values measured on day 4.

[00058] **Bioavailability - serum measurements.** 1 mg resveratrol doses of R-P 104 or resveratrol in Neobee M5 oil (triglyceride of coconut oil, Spectrum Chemical Manufacturing Corp., Gardena, CA, USA) were administered to athymic mice by oral gavage, injections into the peritoneum (IP) or intra-venous (IV) via the tail vein. Two mice were used per condition. Blood was extracted from the axillary vessels at appropriate times before the mice were euthanized. Serum was isolated and measurements were carried out using standard procedures (Semple-Rowland and Van der WeI, 1992). Briefly, serum from two mice were combined and divided into two equal parts. 10,000 U/mL beta-glucuronidase (Type IX-A, *E.Coli*, Sigma) and 50µl sulfatase (Type VI, *Aerobacter aerogenes*, Sigma) were added to one part sample. Both parts were incubated at 37°C for 5 hours and were then extracted with 2 x 0.5 mL ethyl acetate. Upper phases were mixed and the solvent was evaporated. The residual pellets were dissolved in 0.1mL HPLC solvent B. These samples were then analyzed by the HPLC method to quantitate resveratrol concentrations in the serum samples. As an additional control, resveratrol-spiked serum was analyzed and found to be extracted with 90% efficiency.

[00059] **Animal studies.** Twenty one female athymic nu/nu, 5 to 6 weeks-old mice were each given a dorsal subcutaneous injection of SK-N-AS (3x10⁶) neuroblastoma cells suspended in 500 µl of 1:1 culture medium and basement membrane matrix suspension (BD Matrigel; Fisher Scientific, Pittsburgh, PA, USA). Tumors were allowed to grow for 4 days before the randomization of the animals into 4 groups of 5 animals each. Tumors were allowed to grow to approximately 200 mm³ before starting administration of 5 injections of 200 µl R-P 104 (approximately 5mg resveratrol) or vehicle over a period of two weeks either in the tissue adjacent to the tumor (peri-tumor) or into the
tumor (intra-tumor). Tumor volume was measured twice a week in three dimensions with calipers and the volume was approximated by multiplying the three measurements.

On the day after the last dose, the animals were euthanized, photographed, their tumors were harvested, measured in three dimensions using calipers, fixed in 10% neutral buffered formalin and processed for histology. Five-micrometer sections were cut in a masked fashion to obtain sections that encompassed the full circumference of the tumor that were then stained with Hematoxylin-Eosin (H&E). These were examined microscopically and the histopathologic features were recorded as previously described elsewhere (Albert et al, 2004, Grostern et al, 2002, Sabet et al, 1999). Histopathology and tumor section evaluations were carried out independently by two trained observers (DMA and PvG). The percent of the tumor that was necrotic (percent necrotic area) in R-P 104- and vehicle-treated tumors were determined in H&E stained tumor sections. The outline of the tumor in each section was traced from a microscopically digitized image, and the areas of viable and non-viable appearing tumor were measured using ImageJ software (Wayne Rasband, NIH, rsbweb freeware).

Statistical analysis. All data were analyzed using one-way ANOVA. If this initial analysis found a significant difference among the groups, then t tests were used to test for pairwise differences between groups. AU responses were transformed to the log scale to obtain data, which satisfy the assumptions required for ANOVA. Means and standard errors (SE) were calculated by transforming back from the log scale. Differences were considered significant at $P < 0.05$.

Results: Solubility, preparation and characterization of R-P 104. As previously stated, resveratrol is a hydrophobic compound that is sparingly soluble in water. Improvement in its solubility in aqueous media was attempted using several emulsifiers in combination with either ethanol or DMSO, starting with the commonly used nonionic surfactants, Tween 80 and CremophorEL (van Zuylen et al, 2001). The resveratrol solubilities obtained with various formulations are reported in Table 1 below.
Table 1: Resveratrol Solubility for Various Formulations

<table>
<thead>
<tr>
<th>Emulsifier</th>
<th>Formulation</th>
<th>Resveratrol solubility (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80</td>
<td>4%tween80, 20%DMSO</td>
<td>3.67</td>
</tr>
<tr>
<td></td>
<td>4%tween80, 20%EtOH</td>
<td>3.77</td>
</tr>
<tr>
<td>Cremophor EL</td>
<td>4%CremEL, 20%DMSO</td>
<td>3.30</td>
</tr>
<tr>
<td></td>
<td>4%CremEL, 20%EtOH</td>
<td>3.16</td>
</tr>
<tr>
<td>L62</td>
<td>4%L62, 3%DMSO</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>4%L62, 20%EtOH</td>
<td>0.51</td>
</tr>
<tr>
<td>F127</td>
<td>4.5%F127, 10%DMSO</td>
<td>3.76</td>
</tr>
<tr>
<td></td>
<td>4%F127, 20%DMSO</td>
<td>7.67</td>
</tr>
<tr>
<td></td>
<td>4%F127, 20%EtOH</td>
<td>3.87</td>
</tr>
<tr>
<td></td>
<td>5%F127, 2%DMSO</td>
<td>3.45</td>
</tr>
<tr>
<td></td>
<td>10%F127, 2%EtOH</td>
<td>6.10</td>
</tr>
<tr>
<td>P104</td>
<td>4%P104, 20%DMSO</td>
<td>3.58</td>
</tr>
<tr>
<td></td>
<td>5%P104, 2%DMSO</td>
<td>5.38</td>
</tr>
<tr>
<td></td>
<td>4%P104, 20%EtOH</td>
<td>3.58</td>
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<tr>
<td></td>
<td>5%P104, 2%DMSO</td>
<td>5.38</td>
</tr>
<tr>
<td></td>
<td>10%P104, 2%EtOH</td>
<td>19.45</td>
</tr>
</tbody>
</table>

[00064] Excess resveratrol was added in each case and after centrifugation, the resulting resveratrol concentration in the clear supernatant was determined by reverse-phase HPLC. Tween 80 and Cremophor EL micelles were unable to significantly increase the water solubility of resveratrol as was the case for Pluronic® L62, a relatively hydrophobic surfactant. By contrast, the Pluronics® - F127 and P104, resulted in better solubility.

[00065] Surprisingly, of the surfactants tested, 10% Pluronic® P104 with minimum ethanol (2%) solubilized resveratrol much more effectively than the other formulations, and this formulation was selected for further evaluation. The micelles of this formulation were filtered through a 0.2 μm filter to obtain a clear, pale yellow, easily pipettable solution (R-P 104) that could be readily diluted in water, saline or glucose solutions.
The particle size distribution of the resultant micelles was measured. The R-P 104 solution had 90% particle size distribution of less than 107nm in size and a mean size of 65nm (volume weighted) with standard deviation (SD) +/- 30.7nm (see Figure 1).

Using this method of producing the R-P104 solution formulation, concentrations of 20 to 30 mg resveratrol/mL were consistently achieved. Resveratrol in the R-P 104 formulation remained in solution for up to three weeks when stored at 4°C.

In vitro cell viability. We have previously shown in several tumor cell lines that a stock solution of resveratrol in DMSO, when diluted in aqueous media, inhibits cell division and causes apoptosis (Sareen et al, 2006, Sareen et al, 2007, van Ginkel et al, 2007, van Ginkel et al, 2008). R-P104 had an effect similar to resveratrol in DMSO on the viability of several tumor cell lines that were tested. Figure 2 shows the results of cell viability assays of SK-N-AS neuroblastoma cells in the presence of various resveratrol and vehicle formulations. As seen in Figure 2, the viability of cells treated with both resveratrol in DMSO solutions and R-P104 formulations was much reduced as compared to the cells that were either untreated or treated with vehicle formulations.

IC₅₀ values for both the resveratrol in DMSO and the P104 formulations were 50 to 55μM in SK-N-AS neuroblastoma cells. Similar IC₅₀ values were obtained in tests with the uveal melanoma cell lines C918 and Mum2B (data not shown). Thus the data so far suggests that the presence of P104 does not diminish the efficacy of resveratrol in vitro and does not contribute to the cytotoxicity of SK-N-AS cells.

Bioavailability. A soluble formulation of resveratrol allows for intravenous (IV) as well as oral and intra-peritoneal (IP) delivery. The demonstrated in vivo effects of resveratrol despite low serum levels has been an enigma discussed in many publications (Asensi et al, 2002, Baur and Sinclair, 2006, Gescher and Steward, 2003, Gescher, 2008, Kundu and Surh, 2008, Walle et al, 2004). To test the bioavailability of resveratrol with R-P104, we measured the amount of total resveratrol, which includes unmodified as well as the glucuronidated and sulfated metabolites, in mouse serum at different times after a 1mg dose administered either by oral gavage or injected either in the peritoneum (IP) or tail vein (IV). The results are shown in Table 2 below.
Table 2: Recovery from lmg R-P104 dose: Serum levels of total resveratrol

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Oral (µM)</th>
<th>IP (µM)</th>
<th>IV (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>47.1</td>
<td>48.5</td>
<td>113.2</td>
</tr>
<tr>
<td>15</td>
<td>40.8</td>
<td>42.6</td>
<td>89.4</td>
</tr>
<tr>
<td>30</td>
<td>15.1</td>
<td>21.5</td>
<td>7.6</td>
</tr>
<tr>
<td>60</td>
<td>4.8</td>
<td>17.5</td>
<td>3.6</td>
</tr>
</tbody>
</table>

[00071] As reported in previous publications (Baur and Sinclair, 2006), peak serum levels were reached within 5 minutes, irrespective of the route of delivery. Oral gavage and IP administration resulted in similar initial serum levels of resveratrol; however, serum levels persisted longer with IP doses. IV tail vein delivery, however, resulted in more than twice the peak serum levels achieved by the other two routes. Fifteen minutes after dosing, serum levels were still high for IV delivery but dropped rapidly thereafter such that at 60 minutes, serum levels were similar to those from oral delivery. Therefore, by providing resveratrol in a soluble form for IV delivery it is possible to achieve increased serum levels of resveratrol. Further, rapid clearance would suggest that this benefit might be achieved without increasing toxicity.

[00072] Glucuronidated derivatives of resveratrol. In studies measuring serum resveratrol levels in organisms ranging from mice to humans, reported levels generally include both free and metabolized forms of resveratrol. However, direct testing of the biological activity of the metabolites on cancer cells has not been done thus far. Purified formulations of the mono-glucuronidated derivatives of resveratrol shown in Figure 3 (3-OGR) and Figure 4 (4'-OGR) were individually tested on tumor cells. As shown in Figure 5, the viability of SK-N-AS neuroblastoma cells in the presence of both these derivatives indicate that they have no effect at concentrations where free resveratrol causes inhibition of cell growth. No apoptosis was observed even after incubation for more than 5 days at high concentrations (300µM) of the glucuronidated derivatives in neuroblastoma cells (data not shown). Similar findings were obtained with uveal melanoma cell lines that were tested (data not shown). These results suggest that the non-metabolized form of resveratrol produces the significant anti-tumor cytotoxic effects attributed to resveratrol.

[00073] Free resveratrol in serum. Previous studies on tumor suppression in mouse xenograft models used daily oral delivery of 1.0 mg resveratrol as an oil-based formulation (Sareen et al, 2007, van Ginkel et al, 2007, van Ginkel et al, 2008). Our in vitro studies suggest that levels of free resveratrol, and not the metabolites, might be more relevant to the efficacy of resveratrol as an anti-
tumor agent. The levels of resveratrol in serum at different time points after a 1.0 mg dose of R-P 104 delivered by oral, IP and IV (tail vein) administration were measured. Similar measurements were made for oral and IP delivery of the Neobee M5® oil-based formulation. Free resveratrol values obtained are presented in Table 3.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Oral</th>
<th>IP</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neobee (µM)</td>
<td>R-P104 (µM)</td>
<td>Neobee (µM)</td>
</tr>
<tr>
<td>5</td>
<td>9.4</td>
<td>3.7</td>
<td>6.7</td>
</tr>
<tr>
<td>15</td>
<td>0.66</td>
<td>1.72</td>
<td>0.66</td>
</tr>
<tr>
<td>30</td>
<td>0.53</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>60</td>
<td>0.70</td>
<td>0.18</td>
<td>0.70</td>
</tr>
</tbody>
</table>

[00074] In all cases peak values were reached within 5 minutes. Li the case of oral delivery, even if the oil-emulsion resulted in slightly elevated peak levels, by 15 minutes, serum levels were close to the baseline value of 0.5µM. However, with IP delivery, even though peak levels were similar for the two formulations, circulating resveratrol was present in the serum longer with R-P104. IV delivery provided approximately two-fold higher peak resveratrol concentration than either oral or IP routes. Further, serum resveratrol levels were significantly higher with IV than oral delivery for up to 30 minutes post-dosing.

[00075] *In vivo efficacy.* A preliminary dose response test of resveratrol in DMSO (1, 5, 10 and 20mg per dose) delivered locally, was done in a mouse xenograft model of human neuroblastoma (van Ginkel *et al.,* 2007). The drug was injected multiple times in the tissues adjacent to the tumor (peri-tumor). Anti-tumor effects were observed at 5 mg and higher doses with 10 mg resulting in optimum tumor inhibition (76%, P = 0.024). However, eschar formed on the skin at the drug/vehicle injection site, apparently caused by the DMSO vehicle.

[00076] To test whether solubilization in the block co-polymer would reduce these undesirable side-effects while maintaining the positive tumor inhibition, we repeated the experiment using SK-N-AS cells in athymic mice. Tumors were allowed to grow to an approximate volume of 200 mm³ before starting a series of 5 peri-tumor injections, each of 200µL (approx. 5.0 mg resveratrol) R-P104 or vehicle. This was the maximum achievable dose based on the solubility of resveratrol in R-P104 and the lowest dose required based on the dose response study in DMSO.
Figure 6A is a graph of mean tumor size in each group over the course of the study and allows for a comparison of the rate of growth of the tumors in the two groups. Resveratrol-treated mice showed tumor inhibition of about 88% compared to vehicle-treated controls ($P = 0.003$). The average tumor volumes of the treated group after 5 doses of resveratrol was 145.8 mm$^3$ (SE = 54.13 mm$^3$) while the control group tumors grew to an average volume of 1195.4 mm$^3$ (SE = 506 mm$^3$). Further, the skin of the control or treated mice showed no eschar (Figure 6B, left and right panels).

Tumor sections were H&E-stained and viable areas (intact cells with large pleomorphic nuclei that stained predominantly with hematoxylin) were measured with imageJ software. Analysis of the tumor sections from the peri-tumor injected mice revealed that both drug- and vehicle-treated groups of mice contained extensive areas of necrosis (decomposing cells showing eosinophilic staining -Figures 7A, B). This appearance is typically associated with rapidly growing tumors that have outstripped their blood supply. However, in addition to the smaller tumor size in the treated group, on average only 35% of the tumor (21 to 49%) appeared viable compared to 68% (60 to 73%) in the vehicle treated controls indicating that there was increased cell death related to treatment. Microscopic evaluation showed areas of apoptotic cells with pyknotic nuclei in the drug-treated tumors.

Injection of resveratrol directly into a tumor could also be used as a method to de-bulk tumors, especially at inoperable sites. Previous attempts at carrying out this study with resveratrol in DMSO resulted in immediate toxic fatality to the mice irrespective of the presence of resveratrol. The P104 formulation made it possible to deliver the drug intra-tumor. Therefore, a similar experiment to the one outlined above was carried out where 5 mg doses of R-P104 were directly injected into the tumor (intra-tumor). Resveratrol-treated mice showed 72% inhibition of tumor growth compared to vehicle-treated controls ($P = 0.0036$). Unlike previously seen with DMSO, there was no fatality, however, there was some toxicity associated with multiple injections of both the vehicle and drug resulting in weight loss in the mice.

Tumor sections from intra-tumor treatment were H&E-stained and suggested improvement over peri-tumor injections with an even smaller percentage of viable areas in the treated tumors (Figures 7 C and D). On average only about 8% (0 to 25%) of the tumor was viable in R-P104 treated tumors compared to 55% (30 to 69%) in the vehicle-treated tumors when viable areas were quantified using imageJ software on the H&E-stained tumor sections. Microscopic evaluation shows extensive areas of necrosis with ghost cells and liquified cell material interspersed with apoptotic cells with pyknotic nuclei in the drug-treated tumors that are absent in the vehicle-
treated tumors. Therefore, even though tumor volume measurements showed inhibition of tumor growth in both the peri-tumor and intra-tumor models, the histopathology evaluation of the residual tumors suggested the potential for tumor regression and increased potency with intra-tumor injections.

**Discussion:**

Because most effective therapies available for the treatment of advanced stages of cancer are associated with severe side-effects, there is a need for effective, nontoxic drugs. We have previously shown that resveratrol is a non-toxic drug with potent inhibitory effect on tumor growth in animal models of human neuroblastoma, uveal melanoma and breast cancer (Sareen *et al.*, 2007, van Ginkel *et al.*, 2007, van Ginkel *et al.*, 2008). However, due to rapid clearance the systemic levels of resveratrol after oral delivery are very low. Bolus experiments suggest that providing high local levels of resveratrol increases selective cell death in tumors allowing for regression, rather than just tumor growth inhibition. A more soluble form of resveratrol is desirable to increase options for delivery that might enhance the bioavailability of this drug and thereby improve efficacy.

Various surfactants were tested for their ability to solubilize resveratrol. The block copolymers, such as Pluronic® P104, form micelles that may function to not only solubilize lipophilic drugs but may also alter the pharmacokinetics and biodistribution, either by acting as solubilizers or actual drug carriers (Kabanov *et al.*, 2003). Moreover, several of these polymeric micelle-based drug delivery systems have been approved by the FDA (Croy and Kwon, 2004).

The R-P 104 formulation was found to be stable over several weeks and to improve resveratrol’s solubility in aqueous media at least 1000-fold without affecting its *in vitro* efficacy against tumor cells. More importantly it allows for multiple delivery modes, including IV, where higher peak serum unmodified resveratrol concentrations were achieved when compared with oral or IP doses. The low bioavailability of resveratrol has been a source of concern in animal experiments (Walle *et al.*, 2004). The reported serum levels have combined both modified and free resveratrol. The metabolites detected in serum include the sulfated and glucuronidated derivatives of resveratrol (Miksits *et al.*, 2005, Yu *et al.*, 2002) with groups reporting the detection of the mono-glucuronidated derivatives in humans (Boocock *et al.*, 2007, Urpi-Sarda *et al.*, 2007).

By synthesizing the two possible mono-glucuronidated derivatives and testing them *in vitro* on multiple tumor cell types, we have found both these derivatives to be inactive compared to resveratrol even at ten fold higher concentrations. This accentuates the importance of alternate modes and forms of delivery that will lead to higher levels of unmodified resveratrol. In addition,
applications for resveratrol span several therapeutic categories including heart disease (Das and Maulik, 2006), ageing (Labinskyy et al, 2006), infectious diseases (Wang et al, 2004) and metabolic diseases (Elliott and Jirousek, 2008), with each potentially requiring different dosages and delivery routes. R-P 104 is an important first step in providing a soluble formulation that should increase options for resveratrol delivery.

[00086] In the human neuroblastoma model tested, boosting local concentrations by peri-tumor bolus injections significantly inhibited tumor growth, while histology suggested that intra-tumor injections was associated with increased necrosis and apoptosis within the residual, small tumors. Earlier studies with resveratrol in DMSO showed that the drug does not cause damage to surrounding deep, normal tissues (van Ginkel et al, 2007), although the vehicle caused minor skin ulceration. There are indications that the lower dose of R-P 104 did elicit similar results as resveratrol in DMSO without developing eschar at the injection sites. It seems likely that the toxicity observed with intra-tumor injections may be eliminated and significant efficacy maintained by further lowering the dose. Moreover, literature suggests that purification of commercially available pluronics could reduce their toxicity (Bentley et al, 1989, Lowe et al, 1995). These methods are currently being evaluated.

[00087] Our current results indicate that higher availability of free resveratrol at the tumor site increases its therapeutic efficacy. The rapid clearance observed in the bioavailability studies also suggest that there may be advantages to sustained release or steady infusion methods that would maintain a basal level of free resveratrol. A formulation such as R-P 104 allows us the versatility to test these different approaches. This is the first time that a naturally occurring polyphenol has been solubilized by block co-polymer micelles wherein the resulting formulation has been shown to be effective in treating a disorder in an animal disease model. There are several lipophilic non-toxic compounds that have poor solubility in water and could benefit from a similar approach, leading to potentially improved bioavailability, efficacy and versatile delivery options.

[00088] While the present invention has been described in what is perceived to be the most practical and preferred embodiments and examples, it is to be understood that the invention is not intended to be limited to the specific embodiments set forth above. Rather, it is recognized that modifications may be made by one of skill in the art of the invention without departing from the spirit or intent of the invention and, therefore, the invention is to be taken as including all reasonable equivalents to the subject matter of the appended claims. All references cited herein are incorporated by reference for all purposes.
References:


-19-


WE CLAIM:

1. An aqueous solution, the solution comprising:
   \( \text{trans-3,4',5-trihydroxystilbene; } \)
   water; and
   an effective amount of a solubilizer comprising poloxamer 334, wherein the solubilizer renders the \( \text{trans-3,4',5-trihydroxystilbene water soluble. } \)

2. The solution of claim 1, wherein the poloxamer 334 makes up between 5% and 20% of the solution by weight.

3. The solution of claim 2, wherein the poloxamer 334 makes up about 10% of the solution by weight.

4. The solution of any one of claims 1-3, wherein the solution additionally comprises ethanol.

5. The solution of any one of claims 1-4, wherein the concentration of \( \text{trans-3,4',5-trihydroxystilbene } \) in the solution is between 20 mg/mL and 30 mg/mL.

6. The solution as described in any one of claims 1-5 for the treatment of cancer.

7. The solution of claim 6, wherein the cancer to be treated is neuroblastoma, breast cancer, cutaneous melanoma or uveal melanoma.

8. A method of treating cancer, the method comprising the step of administering to a patient having cancer any of the solutions of claims 1-5, and wherein the amount of solution administered is sufficient to reduce cancer symptoms.

9. The method of claim 8, wherein the solution is administered by intravenous injection, intraperitoneal injection, intratumor injection, peri-tumor injection or orally.
10. The method of claim 8 or 9, wherein the cancer treated is neuroblastoma, breast cancer, cutaneous melanoma or uveal melanoma.

11. A method of making an aqueous solution wherein \textit{trans}-3,4',5-trihydroxystilbene is rendered water soluble, the method comprising the step of mixing \textit{trans}-3,4',5-trihydroxystilbene, water, and poloxamer 334 to make a solution.

12. The method of claim 11, wherein the amount of poloxamer 334 mixed in to make the solution is from 5-20% of the total mixture by weight.

13. The method of claim 12, wherein the amount of poloxamer 334 mixed in to make the solution is about 10% of the total mixture by weight.

14. The method of any one of claims 11-13, wherein ethanol is mixed with the \textit{trans}-3,4',5-trihydroxystilbene, water, and poloxamer 334 to make the solution.

15. The method of claim 14, wherein the step of mixing ethanol, \textit{trans}-3,4',5-trihydroxystilbene, water, and poloxamer 334 to make the solution includes the steps of: mixing the ethanol and \textit{trans}-3,4',5-trihydroxystilbene to make a first solution; mixing the water and the poloxamer 334 to make a second solution; and mixing the first and second solutions to make the aqueous solution.

16. The method of any one of claims 11-14, wherein the amount of \textit{trans}-3,4',5-trihydroxystilbene added to the mixture is in excess of 30 mg \textit{trans}-3,4',5-trihydroxystilbene per mL of mixture.

17. The method of claim 16, comprising the additional step of centrifuging the mixture and removing the solution as the supernatant liquid.

18. The method of claim 16 or 17, comprising the additional step of passing the solution through a filter.
19. The method of claim 18, wherein the filter has a pore size of about 0.2µm.

20. A method of making a medicament solution for the treatment of cancer, the method comprising the step of mixing grøn,s-3,4',5-trihydroxystilbene, water, and poloxamer 334 to make a solution, and wherein the solution is effective for reducing cancer symptoms when administered to a patient having cancer.

21. The method of claim 20, wherein the amount of poloxamer 334 mixed in to make the solution is from 5-20% of the total mixture by weight.

22. The method of claim 21, wherein the amount of poloxamer 334 mixed in to make the solution is about 10% of the total mixture by weight.

23. The method of any one of claims 20-22, wherein ethanol is mixed with the trans-3,4',5-trihydroxystilbene, water, and poloxamer 334 to make the solution.

24. The method of any one of claims 20-23, wherein the medicament is for the treatment of neuroblastoma, breast cancer, retinoblastoma, cutaneous melanoma, or uveal melanoma.
Fig. 1
Fig. 2
3-OGR: 3-O-Glucuronidated Resveratrol

Fig. 3
4'-OGR: 4'-O-Glucuronidated Resveratrol

Fig. 4
Fig. 5
A.

![Graph with data points showing the comparison between Vehicle and 5mg R-P104 treatments over 25 days.](image)

B.

![Images showing tumor growth over time.](image)

**Fig. 6**
A  CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01 N 31/00; A61 K 31/045 (201 0.01 )
USPC - 514/730

According to International Patent Classification (IPC) or to both national classification and IPC

B  FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC - 514/730

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/473, 780, 514/25, 27, 422, 729, 733, 748, 751, 754, 756, 764-765, 536/4 1, 548/525 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB, USPT, EPAB, JPAJ), Google, Google Scholar
Search Terms Used: resveratrol, trans-3,4',5'-trihydroxystilbene, trans-RV, poloxamer 334, pluronic 104, polyoxypropylene-

polyoxyethylene non-ionic block copolymers, ethanol, water, aqueous solution, solubility, cancer, solubilizer, lipophilic

C  DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
</table>

Date of the actual completion of the international search
07 January 2010 (07 01 2010)

Date of mailing of the international search report
01 FEB 2010

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No 71-273-0201

Authorized officer
Lee W Young
PCT Helpdesk, 571 272-4300
PCT OSP 571 272 7774

Form PCT/ISA/2 (second sheet) (July 2009)
**INTERNATIONAL SEARCH REPORT**

**Box No. 11 Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **Claims Nos**
   - because they relate to subject matter not required to be searched by this Authority, namely

2. **Claims Nos**
   - because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically

3. **Claims Nos 5-10, 16-19 and 24**
   - because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6(4(a))

**Box No. 11 Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. **As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims**

2. **As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees**

3. **As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos**

4. **No required additional search fees were timely paid by the applicant Consequently, this international search report is restricted to the invention first mentioned in the claims, it is covered by claims Nos**

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation
- No protest accompanied the payment of additional search fees

Form PCT/ISA/2 10 (continuation of first sheet (2)) (July 20Q9)